

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
8 December 2011 (08.12.2011)

(10) International Publication Number
WO 2011/151723 A2

- (51) International Patent Classification: Not classified
- (21) International Application Number: PCT/IB20 11/001542
- (22) International Filing Date: 1 June 2011 (01.06.2011)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:

61/396,719	1 June 2010 (01.06.2010)	US
61/465,405	18 March 2011 (18.03.2011)	US
- (71) Applicant (for all designated States except US): **NOVARTIS AG** [CH/CH]; Lichtstrasse 35, CH-4056 Basel (CH).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **KOMMAREDDY, Sushma** [US/US]; Novartis Vaccines, 45 Sidney Street, Cambridge, MA 02139 (US). **SCAMPINI, Amanda** [US/US]; Novartis Vaccines, 45 Sidney Street, Cambridge, MA 02139 (US). **BAUDNER, Barbara** [IT/IT]; Novartis Vaccines, Via Fiorentina, 1-53100 Siena (IT). **O'HAGAN, Derek** [US/US]; Novartis Vaccines, 45 Sidney

Street, Cambridge, MA 02139 (US). **SINGH, Manmohan** [US/US]; Novartis Vaccines, 45 Sidney Street, Cambridge, MA 02139 (US).

(74) Agents: **MARSHALL, Cameron, John** et al; Carpmaels & Ransford, One Southampton Row, London WC1B 5HA (GB).

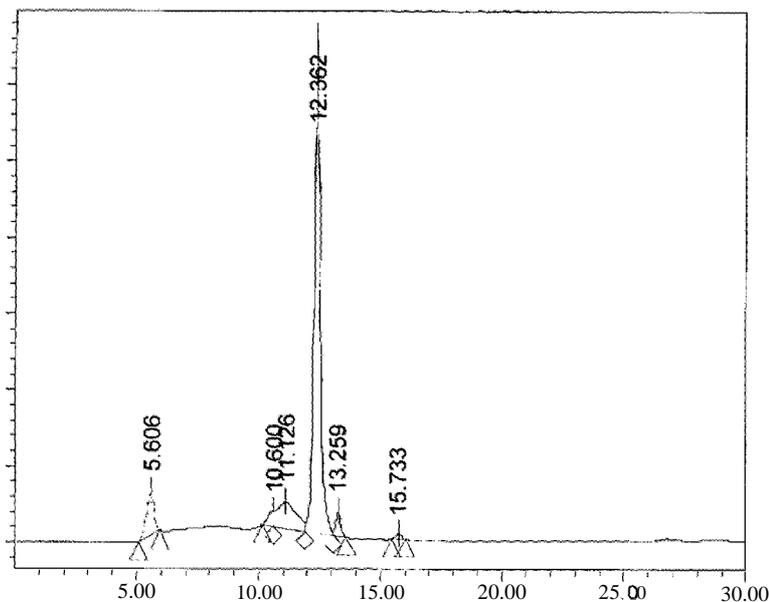
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,

[Continued on next page]

(54) Title: CONCENTRATION OF VACCINE ANTIGENS WITHOUT LYOPHILIZATION

FIGURE 1C



(57) Abstract: An antigen concentration procedure does not involve lyophilisation of a bulk antigen before its final formulation and/or delivery. Thus a process for preparing a vaccine comprises steps of (i) increasing the concentration of an antigen in a liquid composition including that antigen, to provide a concentrated antigen, and (ii) formulating a vaccine from the concentrated antigen. The concentrated antigen is not lyophilised between or during steps (i) and (ii). The invention is particularly useful for preparing solid vaccine forms.

Wo 2011/151723 A2

SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG). **Published:**

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

CONCENTRATION OF VACCINE ANTIGENS WITHOUT LYOPHILIZATION

This application claims the benefit of US provisional applications 61/396,719 (filed June 1st 2010) and 61/465,405 (filed March 18th 2011), the complete contents of both of which are hereby incorporated herein by reference for all purposes.

5 TECHNICAL FIELD

This invention is in the field of processing antigen solutions for use in vaccines,

BACKGROUND ART

During vaccine manufacture it is often the case that the concentration of antigen in a manufacturing bulk exceeds the concentration in a final patient formulation, and so the process involves a step in which the bulk is diluted. In some situations, however, it is necessary to increase the antigen concentration in an aqueous bulk, and the invention concerns processes for concentrating antigens. Useful processes should increase an antigen's concentration without destroying its immunogenicity.

One situation where antigen concentration is required is for new delivery techniques where only a small volume of material is delivered. For instance, vaccines can be delivered by microneedles [2,3] or by thin films or strips [1,14-17], These techniques deliver much less volume than the typical intramuscular injection of 0.5ml but they may require the same amount of antigen, which will often require a more concentrated bulk antigen.

One existing concentration process which can increase the concentration of an individual influenza virus hemagglutinin (HA) from 125-500pg/ml to 14mg/ml involves tangential flow filtration (TFF) of a starting volume of aqueous material to a concentration of 10mg/ml, then lyophilisation, then reconstitution of the lyophilisate in a smaller aqueous volume than the starting volume. This process can be performed on three different monovalent HA bulks, and their reconstitution as a single trivalent aqueous composition can provide a final HA concentration of 42mg/ml.

It is an object of the invention to provide further and improved processes for increasing the concentration of antigen in a material for use in vaccine manufacture, and particularly for influenza vaccine manufacture, such as influenza vaccines which are not delivered by intramuscular injection.

DISCLOSURE OF THE INVENTION

In contrast to an existing process in which antigen concentration relies on lyophilisation of a large aqueous volume followed by reconstitution of lyophilisates in a smaller aqueous volume, the invention provides an antigen concentration procedure which does not involve lyophilisation of a bulk antigen before its final formulation and/or delivery. The inventors have found that, although the lyophilisation procedure can enhance the shelf-life of an antigen while achieving the desired concentration, it can damage the antigen such that the amount of functional antigen which is recovered post-reconstitution is lower than expected. Moreover, lyophilised antigen often does not function well in standard assays which have been established using soluble antigens (e.g. the SR1D

assay for hemagglutinin quantification). A lyophilisation-free concentration procedure thus improves the final vaccine product, permits the use of standard assays, and can also avoid the need to add lyoprotectants (such as sucrose) to the final vaccine.

Thus the invention provides a process for preparing a vaccine, comprising steps of (i) increasing the concentration of an antigen in a liquid composition including that antigen, to provide a concentrated antigen, and (ii) formulating a vaccine from the concentrated antigen; wherein the concentrated antigen is not lyophilised between or during steps (i) and (ii).

The invention also provides a vaccine prepared by this process. The invention is particularly useful for preparing solid vaccine forms.

10 The invention also provides a process for preparing an influenza vaccine, comprising steps of (i) increasing the concentration of an influenza virus hemagglutinin in a liquid composition including that hemagglutinin, to provide a concentrated antigen, and (ii) formulating an influenza vaccine from the concentrated antigen; wherein the concentrated antigen is not lyophilised between or during steps (i) and (ii).

15 The invention also provides a process for preparing a vaccine comprising hemagglutinin from n different influenza virus strains, comprising steps of (i) increasing the concentration of influenza virus hemagglutinin in n separate liquid compositions, each including hemagglutinin from a different strain, to provide n separate concentrated antigens, and (ii) formulating a multivalent influenza vaccine from the n separate concentrated antigens; wherein none of the n separate concentrated
20 antigens is lyophilised between or during steps (i) and (ii). The value of n is 2 or more (*e.g.* 2, 3, 4, or 5) but will typically be 3 (I.e. a trivalent influenza vaccine) or 4 (*i.e.* a tetravalent influenza vaccine).

The invention also provides a bulk liquid vaccine comprising influenza virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least 12mg/ml and (b) the vaccine is substantially free from sucrose.

25 The invention also provides a bulk liquid vaccine comprising hemagglutinin from at least two strains of influenza virus, wherein (a) the hemagglutinin concentration is at least 2mg/ml/strain and (b) the vaccine is substantially free from sucrose.

The antigen

The invention is useful for concentrating antigens from various sources. The antigen may be from a
30 bacterium, a virus, a fungus, or a parasite. Thus the vaccine may protect against disease caused by a bacterium, a virus, a fungus, and/or a parasite.

Typical bacteria for use with the invention include, but are not limited to:

- ***Bordetella***, such as *B. pertussis*.
- ***Clostridia***, such as *C.letani* and *C.botulinum*
- 35 • ***Corynebacteria***, such as *C.diphtheriae*.

- * *Pasteurella*, such as *Haemophilus influenzae*.
- *Mycobacteria*, such as *M.tuberculosis*, *M.bovis* and the attenuated *Bacillus Calmette Guerin*.
- *Neisseria*, such as *N.meningitidis* and *N. gonorrhoeae*.
- *Salmonella*, such as *S.typhi*, *S.paratyphi*, *S.typhimurium*, *S.enteritidis*.
- 5 • *Streptococci*, such as *S.pneumoniae* (pneumococcus), *S.agalactiae* and *S.pyogenes*.

Typical viruses for use with the invention include, but are not limited to:

- * *Orthomyxovirus*, such as an influenza A, B or C virus. Influenza A or B viruses may be inter pandemic (annual/seasonal) strains, or from strains with the potential to cause a pandemic outbreak (*i.e.*, influenza strains with new hemagglutinin compared to a hemagglutinin in currently circulating strains, or influenza strains which are pathogenic in avian subjects and have the potential to be transmitted horizontally in the human population, or influenza strains which are pathogenic to humans). Depending on the particular season and on the nature of the strain, an influenza A virus may be derived from one or more of the following hemagglutinin subtypes: H1, H2, H3, H4, H5, H6, H7,H8, H9, H10, H11, H12, H13, H14, H15 or H16. More details are given below.
- 10
- ◆ *Paramyxoviridae* viruses, such as Pneumoviruses (RSV), Paramyxoviruses (PIV) and Morbilliviruses (Measles).
- * *Pneumovirus* or *metapneumovirus*, for example respiratory syncytial virus (RSV), Bovine respiratory syncytial virus, Pneumonia virus of mice, and Turkey rhinotracheitis virus.
- 20 Preferably, the Pneumovirus is RSV or human metapneumovirus (HMPV).
- *Paramyxovirus*, such as Parainfluenza virus (PIV) type 1, 2, 3 or 4, Mumps, Sendai viruses, Simian virus 5, Bovine parainfluenza virus and Newcastle disease virus. Preferably, the Paramyxovirus is PIV or Mumps.
- * *Picomavirus*, such as Enteroviruses, Rhinoviruses, Hepamavirus, Cardioviruses and Aphthoviruses. Enteroviruses include Poliovirus types 1, 2 or 3, Coxsackie A virus types 1 to 22 and 24, Coxsackie B virus types 1 to 6, Echovirus (ECHO) virus) types 1 to 9, 11 to 27 and 29 to 34 and Enterovirus 68 to 71. Preferably, the Enterovirus is poliovirus *e.g.* a type 1 strain such as Mahoney or Brunenders, a type 2 strain such as MEF-1, or a type 3 strain such as Saukett. An example of a Heparnaviruses (also named Hepatoviruses) is Hepatitis A virus.
- 25
- * *Togavirus*, such as a Rubivirus, an Alphavirus, or an Arterivirus. Rubiviruses, such as Rubella virus, are preferred. Useful alphaviruses for inactivation include aquatic alphaviruses, such as salmon pancreas disease virus and sleeping disease virus.
- 30
- *Flavivirus*, such as Tick-borne encephalitis (TBE), Dengue (types 1, 2, 3 or 4), Yellow Fever, Japanese encephalitis, West Nile encephalitis, St. Louis encephalitis, Russian spring-summer encephalitis, Powassan encephalitis.
- 35

- *Hepatitis C virus (HCV).*
 - *Pestivirus*, such as Bovine viral diarrhea (BVDV), Classical swine fever (CSFV) or Border disease (BDV).
 - *Hepadnavirus*, such as Hepatitis B virus.
 - 5 • *Rhabdovirus*, such as a Lyssavims (e.g. a rabies virus) and Vesiculovirus (VSV).
 - *Caliciviridae*, such as Norwalk virus, and Norwalk-like Viruses, such as Hawaii Virus and Snow Mountain Virus, and Vesivirus, such as Vesicular Exanthema of Swine Virus.
 - *Coronavirus*, such as a SARS, Human respirator) coronavirus, Avian infectious bronchitis (IBV), Mouse hepatitis virus (MHV), and Porcine transmissible gastroenteritis virus (TGEV).
 - 10 • *Retrovirus*, such as an Oncovirus, a Lentivirus or a Spumavirus. An oncovirus may be HTLV-1, HTLV-2 or HTLV-3. A lentivirus may be SIV, HIV-1 or HIV-2.
 - *Reovirus*, such as an Orthoreovirus, a Rotavirus, an Orbivirus, or a Coltivirus.
 - *Parvovirus*, such as Parvovirus B 19, or Bocavirus.
 - *Human Herpesvirus*, such as Herpes Simplex Viruses (HSV), Varicella-zoster virus (VZV),
15 Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus 6 (HHV6), Human Herpesvirus 7 (HHV7), and Human Herpesvirus 8 (HHV8).
 - *Papovaviruses*, such as Papillomaviruses and Polyomaviruses. Papillomaviruses include HPV serotypes 1, 2, 4, 5, 6, 8, 11, 13, 16, 18, 31, 33, 35, 39, 41, 42, 47, 51, 57, 58, 63 and 65.
 - *Adenoviridae*, including any of human adenoviruses A, B, C, D, E, F or G.
- 20 The invention is ideal for preparing vaccines for viruses, and in particular viruses where the vaccine antigen is a viral surface glycoprotein. Thus the invention is ideal for concentrating influenza virus hemagglutinin for preparing influenza vaccines, as described below in more detail. The concentration step can provide an influenza vaccine antigen with a HA content of >5mg/ml, >10mg/ml, >15mg/ml, >20mg/ml, >25mg/ml, and even >30mg/ml,

25 *The liquid composition*

A process of the invention increases the concentration of an antigen in a liquid composition, thereby providing a concentrated antigen for formulation purposes.

A preferred liquid composition is one which has never been lyophilised.

30 A preferred liquid composition is substantially free from lyoprotectants. Thus a composition may be substantially free from exogenous sugar alcohols (in particular: sorbitol, mannitol, maltitol, erythritol, xylitol) and/or from exogenous disaccharides (in particular: sucrose, trehalose, maltose, lactulose, lactose, cellobiose). The combined concentration of (sorbitol, mannitol, maltitol, erythritol,

xylitol, sucrose, trehalose, maltose, lactulose, lactose, cellobiose) in a liquid composition may thus be less than 10mg/ml (*i.e.* less than 1%) and is ideally less than 1mg/ml *e.g.* less than 0.1mg/ml.

A typical liquid composition is a bulk vaccine *e.g.* containing enough antigen for at least 500 separate human unit doses of the vaccine.

- 5 The liquid **composition** may be monovalent (*i.e.* **containing** vaccine antigen for protecting against only one pathogen) or multivalent (*i.e.* containing vaccine antigen for protecting against more than one pathogen, which includes where there is more than one different non-cross-protective pathogen *e.g.* multiple meningococcal **serogroups**. or multiple influenza A virus hemagglutinin types). It is preferably **monovalent**.
- 10 The invention can be used with liquid samples having a variety of vaccine antigen concentrations. Typically the liquid sample will include a vaccine antigen at a concentration of at least 1µg/ml.

The concentration step

A process of the invention involves a step in which the concentration of an antigen is increased. Various techniques can be used for this concentration step including, but not limited to: centrifugal
15 **filtration; ultrafiltration; or tangential flow filtration (also known as crossflow filtration). Preferred methods use TFF as this can achieve good concentration even without requiring lyophilisation.**

Centrifugal filtration involves centrifugation of a liquid through a filter. The filter retains the antigen to be concentrated but does not retain solvent or smaller solutes. As the volume of the filtrate increases, the concentration of the antigen in the retentate also increases. This technique typically
20 **uses a fixed angle rotor. Various suitable centrifugal filtration devices are commercially available e.g. the products sold under trade marks Centricon™, Vivaspin™ and Spintek™. The cut-off of the filter will be selected such that the antigen of interest remains in the retentate.**

Ultrafiltration involves the use of hydrostatic pressure to force a liquid against a semipermeable membrane. The filter retains the antigen to be concentrated but does not retain solvent or smaller
25 **solute. Continued application of hydrostatic pressure causes the volume of the filtrate to increase, and thus the concentration of the antigen in the retentate also increases. Many ultrafiltration membranes are commercially available. The molecular weight cut-off (MWCO) of an ultrafiltration membrane determines which solutes can pass through the membrane (*i.e.* into the filtrate) and which are retained (*i.e.* in the retentate). The MWCO of the filter used with the invention will be selected**
30 **such that substantially all of the antigen of interest remains in the retentate.**

Tangential flow filtration (TFF) involves passing a liquid tangentially across a filter membrane. The sample side is typically held at a positive pressure relative to the filtrate side. As the liquid flows over the filter, components therein can pass through the membrane into the filtrate. Continued flow causes the volume of the filtrate to increase, and thus the concentration of the antigen in the retentate
35 **increases. TFF contrasts with deadend filtration, in which sample is passed through a membrane rather than tangentially to it. Many TFF systems are commercially available. The MWCO of a TFF**

membrane determines which solutes can pass through the membrane (*i.e.* into the filtrate) and which are retained (*i.e.* in the **retentate**). The MWCO of a TFF filter used with the invention will be selected such that substantially all of the antigen of interest remains in the retentate.

These three concentration **techniques** are not **mutually** exclusive *e.g.* the invention can use tangential
 5 **flow ultrafiltration**.

Whichever technique is chosen, it preferably increases the concentration of the antigen of interest by at least n -fold, where n is 5, 6, 7, 8, 9, 10, 12, **15**, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80 or more.

The formulation step

After antigen concentration, a process of the **invention** formulates the concentrated antigen as a
 10 vaccine, without any intermediate **lyophilisation**. The **formulation step** also does not involve **lyophilisation of the antigen**. Ideally, there is also **no post-formulation lyophilisation** *i.e.* the vaccine antigen avoids **lyophilisation** from the start of concentration through to patient administration.

The invention can be used for preparing various vaccine formulations. The use of a concentration
 15 step means that the invention is ideal for techniques which involve the delivery of small volumes of material to a patient. For instance, the invention is useful for preparing liquid vaccine formulations which have a unit dose volume of 0.1ml or less (*e.g.* for intradermal injection). The invention is also useful for preparing solid (but not lyophilised) vaccine formulations, as these can require high antigen concentrations. As described in more detail below, suitable solid formulations include, but are not limited to, solid biodegradable microneedles, coated microneedles, and thin films. Thus the
 20 formulation step in a process of the invention may comprise: preparing a solid vaccine form from the concentrated antigen. This step may involve drying, but not freeze drying *e.g.* it may involve evaporation [11].

Formulated vaccines of the invention are sometimes substantially free from lyoprotectants, although these are often added to formulations for reasons unrelated to lyophilisation (*e.g.* to enhance coating).
 25 In some embodiments, therefore, a formulated vaccine of the invention may be substantially free from sugar alcohols (in particular: sorbitol, mannitol, maltitol, erythritol, xylitol) and/or from disaccharides (in particular: sucrose, trehalose, maltose, lactulose, lactose, cellobiose). The combined concentration of (sorbitol, mannitol, maltitol, erythritol, xylitol, sucrose, trehalose, maltose, lactulose, lactose, cellobiose) in a formulated vaccines may thus be less than 10mg/ml (*i.e.* less than
 30 1%) *e.g.* less than 1mg/ml or less than 0.1mg/ml.

The formulation step ideally takes place within 4 weeks (*e.g.* within 2 weeks) of the concentration step, to ensure that the concentrated antigen retains suitable efficacy

Vaccines of the invention are ideally free from inulin. Also, vaccines of the invention ideally do not include surfactant vesicles which encapsulate viral antigens.

Solid biodegradable microneedles

One useful solid formulation which can be prepared using the invention is a solid biodegradable microneedle. These are typically not administered alone but, rather, multiple needles are administered simultaneously *e.g.* as a skin patch comprising a plurality of microneedles.

- 5 The microneedles are solid, such that they retain their structural integrity during storage and can penetrate a subject's skin when the patch is applied. The mechanical characteristics which are required for skin penetration depend on the organism in question, but they will usually have sufficient strength to penetrate human skin. Materials for forming suitable solid needles are readily available and these can be tested to determine appropriate concentrations *etc.* for any particular need.
- 10 The microneedles are biosoluble and biodegradable. Thus the solid material dissolves in the skin after the patch is applied, in contrast to the coated microneedles used in references 2 & 3 (see below). Having dissolved, the material will then be metabolised to give harmless end-products. The timescale for dissolving after applying the patch can vary, but dissolving will typically commence immediately after applying the patch (*e.g.* within 10 seconds) and may continue for *e.g.* up to 1 minute, 5 minutes,
- 15 10 minutes, 20 minutes, 30 minutes, 1 hour, 5 hours, 10 hours, or 24 hours, until the microneedle has fully dissolved. Materials with suitable *in vivo* dissolving kinetics are readily available and these can be varied and tested to determine appropriate concentrations *etc.* for any desired dissolution profile.

Suitable matrix materials for forming the microneedles will typically be biosoluble and biodegradable polymers, and these may comprise one or more carbohydrates. For example, the

20 material may comprise a cellulose, a dextrin, a dextran, a disaccharide, a chitosan, a chitin, *etc.*, or mixtures thereof. Other GRAS materials may also be used.

Suitable celluloses include, but are not limited to, cellulose, sodium carboxymethyl cellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Suitable

25 dextrins include, but are not limited to, maltodextrin, cyclodextrin, amylopectin, icodextrin, yellow dextrin, and white dextrins. Suitable disaccharides include, but are not limited to, sucrose, lactose, maltose, trehalose, turanose, and cellobiose. One suitable material for forming biosoluble and biodegradable microneedles is a dextrin/trehalose mixture.

The microneedles can penetrate the skin. They should be long enough to penetrate through the epidermis to deliver material into the dermis (*i.e.* intradermal delivery), but are ideally not so long

30 that they can penetrate into or past the hypodermis. They will typically be 100-2500 μm long *e.g.* between 1250-1750 μm long, or about 1500 μm . At the time of delivery the tip may penetrate the dermis, but the base of the needle may remain in the epidermis.

The microneedles can have various shapes and geometries. They will typically be tapered with a skin-facing point *e.g.* shaped as pyramids or cones. A tapered microneedle with a widest diameter of

35 <500 μm is typical.

A single patch will typically include a plurality of microneedles *e.g.* >10, >20, >30, ≥ 40 , >50, >60, >70, >80, >90, >100, >200, >300, ≥ 400 , >50, >750, ≥ 1000 or more per patch. Where a patch includes a plurality of microneedles, it may comprise a backing layer to which all of the microneedles are attached. A unitary backing layer with ≥ 20 projecting microneedles is typical.

5 Where a patch includes a plurality of microneedles, these can be arranged in a regular repeating pattern or array, or may be arranged irregularly.

A patch will typically have an area of 3cm^2 or less, for example $< 2\text{cm}^2$ or $< 1\text{cm}^2$. A circular patch with a diameter of between 0.5cm and 1.5cm is useful.

The density of microneedles on a patch can vary, but may be $\geq 10\text{cm}^{-2}$, $\geq 20\text{cm}^{-2}$, $\geq 30\text{cm}^{-2}$, $\geq 40\text{cm}^{-2}$,
10 $\geq 50\text{cm}^{-2}$, $\geq 60\text{cm}^{-2}$, $\geq 70\text{cm}^{-2}$, $\geq 80\text{cm}^{-2}$ or more.

A patch of the invention has a skin-facing inner face and an environment-facing outer face. The inner face may include an adhesive to facilitate adherence to a subject's skin. When present, it is preferably not present on the microneedles themselves *i.e.* the microneedles are adhesive-free. Rather than have adhesive on the inner face, a patch may have an additional backing which provides an outer adhesive
15 margin for adhering the patch to skin *e.g.* as seen in sticking plasters or nicotine patches.

Patches as described above can be made by following the techniques and guidance in references 4-8. For instance, a mold with 1.5mm-long microneedle cavities can be prepared. A matrix material of dextrin and trehalose can be combined with an influenza vaccine and this aqueous material can be centrifugally cast in the mold to form an array of solid microneedles. A cellulose gel can then be cast
20 over the matrix/vaccine mixture (*e.g.* which mixture has formed a film) to form a backing layer on the patch. When this layer has dried, it can be removed to give a patch from which the solid microneedles project. Thus the formulation step in a process of the invention may comprise: (a) mixing a biosoluble and biodegradable matrix material with the concentrated vaccine antigen; and (b) adding the mixture from step (a) to a mold containing cavities for forming microneedles. It may
25 further comprise: (c) letting the mixture set in the mold, to form solid microneedles; (d) optionally, applying material to the set microneedles to provide a backing layer; and (e) removing the microneedles (and optional backing layer) from the mold.

Patches may be packaged into individual pouches *e.g.* sealed under nitrogen, then heat sealed. They should be stored carefully to avoid damage to the microneedles.

30 **Coated microneedles**

Another useful solid formulation which can be prepared using the invention is a coated microneedle. These are typically not administered alone but, rather, multiple needles are administered simultaneously *e.g.* via a plurality of microneedles. One suitable product is marketed under the trade name of Macroflux™ (Zosano).

35 The microneedles are solid, such that they retain their structural integrity during storage and can penetrate a subject's skin. The mechanical characteristics which are required for skin penetration

depend on the organism in question, but they will usually have sufficient strength to penetrate human skin. The microneedles are solid and remain intact after insertion into a patient's skin (in contrast to the biodegradable microneedles discussed above). Materials for forming suitable solid needles are readily available and these can be tested and selected for any particular need *e.g.* metals (such as stainless steel) or polymers (such as polycarbonate, ideally medical grade). Metal needles can be fabricated by using laser cutting and electro-polishing [9]. Polymer needles can be fabricated by microreplication and/or micromolding (including injection molding). Suitable microneedles are disclosed in references 2, 3, and 9-13.

A concentrated antigen of the invention can be coated onto the microneedles. This coating can be achieved by a simple process such as dip-coating *e.g.* involving a dipping step then a drying step (*e.g.* by evaporation), with repetition as required. Other useful coating techniques are disclosed in reference 11. Thus the formulation step in a process of the invention may comprise: applying the concentrated vaccine antigen to the surface of one or more solid microneedles to provide a coated microneedle device for injection of the vaccine.

A coating solution for applying to the needles can include one or more biosoluble and biodegradable matrix materials, and these may comprise one or more carbohydrates. For example, the material may comprise a cellulose, a dextrin, a dextran, a disaccharide, a chitosan, a chitin, *etc.*, or mixtures thereof. Other GRAS materials may also be used. Suitable celluloses, dextrans and disaccharides are listed above. Thus the formulation step in a process of the invention may comprise: (a) mixing a biosoluble and biodegradable matrix material with the concentrated vaccine antigen; and (b) applying the mixture from step (a) to the surface of one or more solid microneedles to provide a coated microneedle device for injection of the vaccine. Coating may be enhanced by using one or more "deposition enhancing components" as described in reference 11.

The applying steps discussed above may comprise an application sub-step followed by a drying sub-step, and this pair of sub-steps can be performed once or more than once *e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more times.

Microneedles in the device can penetrate the skin when applied. They should be long enough to penetrate through the epidermis to deliver material into the dermis (*i.e.* intradermal delivery), but are ideally not so long that they can penetrate into or past the hypodermis. They will typically be 100-2500 μm long *e.g.* between 250-750 μm long, or about 1500 μm . At the time of delivery the tip may penetrate the dermis, but the base of the needle may remain in the epidermis. The needles can be applied to a patient's skin for between 30 seconds and 30 minutes, and then be removed.

The microneedles can have various shapes and geometries. They will typically be tapered with a skin-facing point *e.g.* shaped as pyramids or cones. A tapered microneedle with a widest diameter of <500 μm is typical.

A microneedle device will typically include a plurality of microneedles *e.g.* >10, >20, >30, >40, >50, >60, >70, >80, >90, >100, >200, ≥ 300 , >400, >50, >750, >1000, ≥ 1500 , ≥ 2000 or more per device (for example, 300-1500 per device). Where a device includes a plurality of microneedles, these will typically all be attached to a unitary backing layer. Where a device includes a plurality of
5 microneedles, these can be arranged in a regular repeating pattern or array, or may be arranged irregularly.

A microneedle device will typically have an area of 3cm^2 or less, for example $<2\text{cm}^2$ or $<1\text{cm}^2$. A circular device with a diameter of between 0.5cm and 1.5cm is useful.

The density of microneedles can vary, but may be $\geq 10\text{cm}^{-2}$, $\geq 20\text{cm}^{-2}$, $\geq 30\text{cm}^{-2}$, $\geq 40\text{cm}^{-2}$, $\geq 50\text{cm}^{-2}$,
10 $\geq 60\text{cm}^{-2}$, $\geq 70\text{cm}^{-2}$, $\geq 80\text{cm}^{-2}$ or more. A device with 2mm between each microneedle, and a density of 14 microneedles/ cm^2 , is useful.

A microneedle device has a skin-facing inner face and an environment-facing outer face. The inner face may include an adhesive to facilitate adherence to a subject's skin. When present, it is preferably not present on the microneedles themselves *i.e.* the microneedles are adhesive-free. Rather than have
15 adhesive on the inner face, a device may have an additional backing which provides an outer adhesive margin for adhering the device to skin.

A microneedle device may be packaged into individual pouches *e.g.* sealed under nitrogen, then heat sealed. They should be stored carefully to avoid damage to the microneedles.

Thin films

20 Another useful solid formulation which can be prepared using the invention is a thin film, such as a thin oral film. These films wet and dissolve quickly upon contact with saliva and buccal tissue, therefore releasing the vaccine antigen in the mouth. The main component of these thin films is typically one or more hydrophilic polymer(s), which can have good mucoadhesive properties to provide strong adhesion to buccal tissue until complete dissolution. Similar films can be used for
25 non-oral delivery *e.g.* for transcutaneous delivery as disclosed in reference 14.

Suitable thin films are typically 10-500 μm thick when initially applied *e.g.* 75-150 μm thick. Their other dimensions can be suitable to fit into a patient's mouth *e.g.* into an adult human mouth or into an infant human mouth.

One suitable type of film is disclosed in reference 15. This film comprises a mucoadhesive bilayer
30 film with (i) Noveon and Eudragit S-100 as a mucoadhesive layer and (ii) a pharmaceutical wax as an impermeable backing layer. Further details of these films are in reference 16.

Another suitable type of film is disclosed in reference 17. This film comprises: (a) one or more water-soluble polymers; (b) one or more mucoadhesive polymers; (c) a vaccine antigen encapsulated within microparticles. Suitable water-soluble polymers include, but are not limited to: pullulan,
35 hydroxypropyl cellulose, polyvinyl pyrrolidone, carboxymethyl cellulose, polyvinyl alcohol, sodium alginate, polyethylene glycol, xanthan gum, tragacanth gum, guar gum, acacia gum, Arabic gum,

polyacrylic acid, methylmethacrylate copolymer, carboxyvinyl polymer, amylase, high amylase starch, hydroxypropylated high amylase starch, dextrin, pectin, chitin, levan, elsinan, collagen, gelatin, zein, gluten, soy protein isolate, whey protein isolate, and casein. Suitable mucoadhesive polymers include, but are not limited to: chitosan, hyaluronate, alginate, poly(acrylic acid),
 5 poly(methacrylic acid), poly(L-lysine), poly(ethyleneimine), poly(ethylene oxide), poly(2-hydroxyethyl methacrylate), and derivatives or copolymers thereof. Useful microparticles are made of a material which releases the particle's encapsulated contents (*i.e.* the vaccine antigen) while still present in the mouth.

The film in reference 14 comprises a cationic poly (P-amino ester) for transcutaneous delivery.

10 An oral film useful with the invention may include a flavouring agent to make the vaccine more palatable during administration.

Thin films can be made a variety of processes, including but not limited to: solvent casting; hot-melt extrusion; solid dispersion extrusion; and rolling.

The formulation step in a process of the invention may comprise: (a) mixing the concentrated
 15 vaccine antigen with one or more orally-soluble polymers; and (b) forming a film using the mixture from step (a) to provide a thin film suitable for buccal administration of the vaccine.

The formulation step in a process of the invention may comprise: (a) mixing the concentrated vaccine antigen with one or more topically-soluble polymers, such as a poly(p-amino ester); and
 20 administration of the vaccine.

These films may be packaged into individual unit dose pouches *e.g.* sealed under nitrogen, then heat sealed. The pouches should be water-tight to keep the films dry during storage.

Methods of treatment, and administration of the vaccine

Formulated vaccines of the invention can be delivered to a subject *e.g.* via their skin, via their buccal
 25 tissue, *etc.* Thus the invention provides a method of raising an immune response in a subject, comprising the step of administering a formulated vaccine of the invention to the subject. This might involve *e.g.* applying a microneedle patch or device to the subject's skin, such that the microneedles penetrate the subject's dermis, or applying a thin film to the subject's buccal tissue or tongue.

The invention also provides a concentrated non-lyophilised antigen for use in a method of
 30 vaccinating a subject. The invention also provides the use of concentrated non-lyophilised antigen in the manufacture of a medicament for raising an immune response in a subject.

Vaccine products are suitable for administering vaccines to human or non-human animal subjects

The immune response raised by these methods and uses will generally include an antibody response, preferably a protective antibody response.

Microneedle patches or devices may be applied to the skin by simple manual application (e.g. as with a sticking plaster or with known skin patches) or may be applied using a spring-driven injector.

Vaccines prepared according to the invention may be used to treat both children and adults.

Treatment can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used
5 in a primary immunisation schedule and/or in a booster immunisation schedule. Multiple doses will typically be administered at least 1 week apart (e.g. about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, etc.).

Influenza vaccination

Processes of the invention are ideal for preparing influenza vaccines. Various forms of influenza
10 virus vaccine are currently available (e.g. see chapters 17 & 18 of reference 18) and current vaccines are based either on inactivated or live attenuated viruses. Inactivated vaccines (whole virus, split virion, or surface antigen) are administered by intramuscular or intradermal injection, whereas live vaccines are administered intranasally. The invention can be used with all of these vaccine forms.

Some embodiments of the invention use a surface antigen influenza vaccine (inactivated). Such
15 vaccines contain fewer viral components than a split or whole virion vaccine. They include the surface antigens hemagglutinin and, typically, also neuraminidase. Processes for preparing these proteins in purified form from influenza viruses are well known in the art. The FLUVIRIN™, AGRIPPAL™ and INFLUVAC™ products are examples of surface antigen influenza vaccines.

Where the invention uses a surface antigen influenza vaccine, this virus may have been grown in
20 eggs or in cell culture (see below). The current standard method for influenza virus growth for vaccines uses embryonated SPF hen eggs, with virus being purified from the egg contents (allantoic fluid). If egg-based viral growth is used then one or more amino acids may be introduced into the allantoic fluid of the egg together with the virus (24). Virus is first grown in eggs. It is then harvested from the infected eggs. Virions can be harvested from the allantoic fluid by various methods. For
25 example, a purification process may involve zonal centrifugation using a linear sucrose gradient solution that includes detergent to disrupt the virions. Antigens may then be purified, after optional dilution, by diafiltration. Chemical means for inactivating a virus include treatment with an effective amount of one or more of the following agents: detergents, formaldehyde, β -propiolactone, methylene blue, psoralen, carboxyfullerene (C60), binary ethylamine, acetyl ethyleneimine, or
30 combinations thereof. Non-chemical methods of viral inactivation are known in the art, such as for example UV light or gamma irradiation.

Some embodiments of the invention can use whole virus, split virus, virosomes, live attenuated virus, or recombinant hemagglutinin. These vaccines can easily be distinguished from surface antigen vaccines by testing their antigens e.g. for the presence of extra influenza virus proteins.

35 Whole inactivated virus can be obtained by harvesting virions from virus-containing fluids (e.g. obtained from eggs or from culture medium) and then treating them as described above.

Split virions are obtained by treating purified virions with detergents (*e.g.* ethyl ether, polysorbate 80, deoxycholate, tri-*N*-butyl phosphate, Triton X-100, Triton N101, cetyltrimethylammonium bromide, Tergitol NP9, *etc.*) to produce **subvirion** preparations, **including** the 'Tween-ether' splitting process. Methods of splitting influenza viruses, for example are well known in the art *e.g.* see refs, 19-24, *etc.*

5 Splitting of the virus is typically carried out by disrupting or fragmenting whole **virus**, whether infectious or non-infectious with a disrupting concentration of a splitting agent. The disruption results in a full or partial **solubilisation** of the **virus proteins**, **altering the integrity of the virus**. Preferred splitting agents are non-ionic and ionic (*e.g.* cationic) surfactants *e.g.* alkylglycosides, alkylthioglycosides, acyl sugars, sulphobetaines, betains, polyoxyethylene-alkylethers, N,N-dialkyl-

10 Glucamides, Hecameg, alkylphenoxy-polyethoxyethanols, NP9, quaternary ammonium compounds, sarcosyl, CTABs (cetyl trimethyl ammonium bromides), tri-*N*-butyl phosphate, myristyltrimethylammonium salts, lipofectin, lipofectamine, and DOT-MA, the octyl- or nonylphenoxy polyoxyethanols (*e.g.* the Triton surfactants, such as Triton X-100 or Triton N101), polyoxyethylene sorbitan esters (the Tween surfactants), polyoxyethylene ethers, polyoxyethylene

15 esters, *etc.* One useful splitting procedure uses the consecutive effects of sodium deoxycholate and formaldehyde, and splitting can take place during initial virion purification (*e.g.* in a sucrose density gradient solution). Thus a splitting process can involve clarification of the virion-containing material (to remove non-virion material), concentration of the harvested virions (*e.g.* using an adsorption method, such as CaHP0₄ adsorption), separation of whole virions from non-virion material, splitting

20 of virions using a splitting agent in a density gradient centrifugation step (*e.g.* using a sucrose gradient that contains a splitting agent such as sodium deoxycholate), and then filtration (*e.g.* ultrafiltration) to remove undesired materials. Split virions can usefully be resuspended in sodium phosphate-buffered isotonic sodium chloride solution. Examples of split vaccines are the BEGRIVAC™, GNTANZA™, FLUARIX™, FLUZONE™ and FLUSHIELD™ products.

25 Virosomes are nucleic acid free viral-like liposomal particles [25]. They can be prepared by solubilization of virus with a detergent followed by removal of the nucleocapsid and reconstitution of the membrane containing the viral glycoproteins. An alternative method for preparing virosomes involves adding viral membrane glycoproteins to excess amounts of phospholipids, to give liposomes with viral proteins in their membrane.

30 Live attenuated viruses are obtained from viruses (grown in eggs or in cell culture), but the viruses are not inactivated. Rather, the virus is attenuated ("att") *e.g.* so as not to produce influenza-like illness in a ferret model of human influenza infection. It may also be a cold-adapted ("ca") strain *i.e.* it can replicate efficiently at 25°C, a temperature that is restrictive for replication of many wildtype influenza viruses. It may also be temperature-sensitive ("ts") *i.e.* its replication is restricted at

35 temperatures at which many wild-type influenza viruses grow efficiently (37-39°C). The cumulative effect of the ca, ts, and att phenotype is that the virus in the attenuated vaccine can replicate in the nasopharynx to induce protective immunity in a typical human patient, but it does not cause disease *i.e.* it is safe for general administration to the target human population. These viruses can be prepared

by purifying virions from virion-containing fluids *e.g.* after clarification of the fluids by centrifugation, then stabilization with buffer (*e.g.* containing sucrose, potassium phosphate, and monosodium glutamate). Live vaccines include the FLUMIST™ product. Although live vaccines can be used with the invention, it is preferred to use non-live vaccines.

- 5 As an alternative to using antigens obtained from virions, haemagglutinin can be expressed in a recombinant host (*e.g.* in an insect cell line, such as Sf9, using a baculovirus vector) and used in purified form [26-28] or in the form of virus-like particles (VLPs; *e.g.* see references 29 & 30).

Some embodiments of the invention use influenza vaccine prepared from viruses which were grown in cell culture, rather than in eggs. When cell culture is used, the viral growth substrate will typically
10 be a cell line of mammalian origin. Suitable mammalian cells of origin include, but are not limited to, hamster, cattle, primate (including humans and monkeys) and dog cells. Various cell types may be used, such as kidney cells, fibroblasts, retinal cells, lung cells, *etc.* Examples of suitable hamster cells are the cell lines having the names BHK21 or HKCC. Suitable monkey cells are *e.g.* African green monkey cells, such as kidney cells as in the Vero cell line. Suitable dog cells are *e.g.* kidney cells, as
15 in the MDCK cell line. Thus suitable cell lines include, but are not limited to: MDCK; CHO; 293T; BHK; Vero; MRC-5; PER.C6; WI-38; *etc.* Preferred mammalian cell lines for growing influenza viruses include: MDCK cells [31-34], derived from Madin Darby canine kidney; Vero cells [35-37], derived from African green monkey (*Cercopithecus aethiops*) kidney; or PER.C6 cells [38], derived from human embryonic retinoblasts. These cell lines are widely available *e.g.* from the American
20 Type Cell Culture (ATCC) collection, from the Coriell Cell Repositories, or from the European Collection of Cell Cultures (ECACC). For example, the ATCC supplies various different Vero cells under catalog numbers CCL-81, CCL-81.2, CRL-I586 and CRL-1587, and it supplies MDCK cells under catalog number CCL-34. PER.C6 is available from the ECACC under deposit number 96022940. As a less-preferred alternative to mammalian cell lines, virus can be grown on avian cell
25 lines [*e.g.* refs. 39-41], including cell lines derived from ducks (*e.g.* duck retina) or hens. Examples of avian cell lines include avian embryonic stem cells [39,42] and duck retina cells [40]. Suitable avian embryonic stem cells, include the EBx cell line derived from chicken embryonic stem cells, EB45, EB14, and EB 14-074 [43]. Chicken embryo fibroblasts (CEF) may also be used.

The most preferred cell lines for growing influenza viruses are MDCK cell lines. The original
30 MDCK cell line is available from the ATCC as CCL-34, but derivatives of this cell line may also be used. For instance, reference 31 discloses a MDCK cell line that was adapted for growth in suspension culture ('MDCK 33016', deposited as DSM ACC 2219). Similarly, reference 44 discloses a MDCK-derived cell line that grows in suspension in serum-free culture ('B-702', deposited as FERM BP-7449). Reference 45 discloses non-tumorigenic MDCK cells, including
35 -MDCK-S' (ATCC PTA-650C), 'MDCK-SF!OT' (ATCC PTA-6501), 'MDCK-SF102' (ATCC PTA-6502) and 'MDCK-SF103' (PTA-6503). Reference 46 discloses MDCK cell lines with high

susceptibility to infection, including 'MDCK.5F1' cells (ATCC CRL-12042). Any of these MDCK cell lines can be used.

Where virus has been grown on a mammalian cell line then products of the invention will advantageously be free from egg proteins (*e.g.* ovalbumin **and** ovomucoid) and from chicken DNA, 5 thereby reducing potential allergenicity.

Hemagglutinin in cell-derived products of the invention can have a different glycosylation pattern from the patterns seen in egg-derived viruses. Thus the HA (**and other glycoproteins**) may **include glycoforms** that are not seen in chicken eggs. Useful HA includes canine **glycoforms**.

The absence of egg-derived materials and of chicken glycoforms provides a way in which vaccine 10 prepared from viruses grown in cell culture can be distinguished from egg-derived products.

Where virus has been grown on a cell line then the culture for growth, and also the viral inoculum used to start the culture, will preferably be free from (*i.e.* will have been tested for and given a negative result for contamination by) herpes simplex virus, respiratory syncytial virus, parainfluenza virus 3, SARS coronavirus, adenovirus, rhinovirus, reoviruses, polyomaviruses, birnaviruses, 15 circoviruses, and/or parvoviruses [47]. Absence of herpes simplex viruses is particularly preferred.

For growth on a cell line, such as on MDCK cells, virus may be grown on cells in suspension [31, 48, 49] or in adherent culture. One suitable MDCK cell line for suspension culture is MDCK 33016 (deposited as DSM ACC 2219). As an alternative, microcarrier culture can be used.

Cell lines **supporting** influenza virus replication are preferably grown in serum-free culture media 20 **and/or protein free media**. A medium is referred to as a serum-free medium in the context of the present invention in which there are no additives from serum of human or animal origin. Protein-free is understood to mean cultures in which multiplication of the cells occurs with exclusion of proteins, growth factors, other protein additives and non-serum proteins, but can optionally include proteins such as trypsin or other proteases that may be necessary for viral growth. The cells growing in such 25 cultures naturally contain proteins themselves.

Cell lines supporting influenza virus replication are preferably grown below 37°C [50] during viral replication *e.g.* 30-36°C, at 31-35°C, or at 33±1 °C.

The method for propagating virus in cultured cells generally includes the steps of inoculating the cultured cells with the strain to be cultured, cultivating the infected cells for a desired time period for 30 virus propagation, such as for example as determined by virus titer or antigen expression (*e.g.* between 24 and 168 hours after inoculation) and collecting the propagated virus. The cultured cells are inoculated with a virus (measured by PFU or TCID₅₀) to cell ratio of 1:500 to 1:1, preferably 1:100 to 1:5, more preferably 1:50 to 1:10. The virus is added to a suspension of the cells or is applied to a monolayer of the cells, and the virus is absorbed on the cells for at least 60 minutes but 35 usually less than 300 minutes, preferably between 90 and 240 minutes at 25°C to 40°C, preferably 28°C to 37°C. The infected cell culture (*e.g.* monolayers) may be removed either by freeze-thawing

or by enzymatic action to increase the viral content of the harvested culture **supernatants**. The harvested fluids are then either inactivated or stored frozen. Cultured cells may be infected at a **multiplicity of infection ("m.o.i.")** of about **0.0001 to 10**, preferably **0.002 to 5**, more preferably to **0.001 to 2**, Still more preferably, the cells are infected at a **m.o.i** of about 0.01. Infected cells may be
5 harvested 30 to 60 hours post **infection**. Preferably, the cells are harvested 34 to 48 hours post **infection**. Still more preferably, the **cells** are harvested 38 to 40 hours **post** infection. Proteases (**typically trypsin**) are generally added during cell culture to allow **viral** release, and the proteases can be added at any **suitable** stage during the **culture**.

A **vaccine product including vaccine prepared** from cell culture preferably contains less than **10ng**
10 (preferably less than **1ng**, and more preferably less than **100pg**) of residual host cell **DNA** per dose, although trace amounts of host cell DNA may be present.

It is preferred that the average length of any residual host cell DNA is less than **500bp e.g.** less than **400bp**, less than **300bp**, less than **200bp**, less than **100bp, etc.**

Contaminating DNA can be removed during **vaccine preparation** using standard **purification**
15 procedures *e.g.* **chromatography, etc.** Removal of residual host cell DNA can be enhanced by **nuclease treatment e.g.** by using a **DNase**. A **convenient method for reducing host cell DNA contamination** is disclosed in references **51 & 52**, involving a two-step treatment, **first using a DNase (e.g. Benzonase)**, which may be used during **viral growth**, and then a **cationic detergent (e.g. CTAB)**, which may be used during **virion disruption**. Treatment with an **alkylating agent**, such as
20 **β-propiolactone**, can also be used to remove host cell DNA, and advantageously may also be used to **inactivate virions** [53].

Some embodiments of the invention use a **monovalent influenza vaccine (i.e. it includes hemagglutinin antigen from a single influenza virus strain)** but in some embodiments it may be a **multivalent vaccine**, such as a **bivalent vaccine, trivalent vaccine, a tetravalent vaccine, or a >4-**
25 **valent vaccine (i.e. including hemagglutinin from more than four different influenza virus strains)**. **Monovalent and multivalent vaccines** are readily distinguished by testing for multiple **HA types**, by **amino acid sequencing, etc.**

A **monovalent vaccine** is particularly useful for immunising against a **pandemic or potentially-pandemic strain**, either during a **pandemic or in a pre-pandemic situation**. Characteristics of these
30 strains are: (a) they **contain a new hemagglutinin** compared to the hemagglutinins in **currently-circulating human strains, i.e. one that has not been evident in the human population for over a decade (e.g. H2)**, or **has not previously been seen at all in the human population (e.g. H5, H6 or H9, that have generally been found only in bird populations)**, such that the human population will be **immunologically naive** to the strain's hemagglutinin; (b) they are **capable of being transmitted**
35 **horizontally in the human population**; and (c) they are **pathogenic to humans**. These strains may have any of influenza A HA subtypes **H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16**. A virus with **H5 hemagglutinin type** is preferred for immunizing against pandemic

influenza, or a H2, H7 or H9 subtype. The invention may protect against one or more of influenza A virus NA subtypes N1, N2, N3, N4, N5, N6, N7, N8 or N9. Thus possible strains include H5N1, H5N3, H9N2, H2N2, H7N1 and H7N7, and any other emerging potentially pandemic strains.

A multivalent vaccine is more typical in a seasonal setting *e.g.* a trivalent vaccine is typical, including hemagglutinins from two influenza A virus strains and one influenza B virus strain, such as from a H1N1 influenza A strain, a H3N2 influenza A virus strain, and an influenza B virus strain. A tetravalent vaccine is also useful [54] *e.g.* including antigens from two influenza A virus strains and two influenza B virus strains, or three influenza A virus strains and one influenza B virus strain. Thus a vaccine may be bivalent, trivalent, tetravalent, *etc.* Except for monovalent vaccines, it is usual to include hemagglutinin from both influenza A and influenza B virus strains. In vaccines including only two influenza A virus strains, these will usually be one H1 strain (*e.g.* a H1N1 strain) and one H3 strain (*e.g.* a H3N2 strain). In some embodiments, however, there may be one pandemic influenza A virus strain and one H1 strain, or one pandemic influenza A virus strain and one H3 strain.

Where a vaccine includes more than one strain of influenza, the different strains are typically grown separately and are mixed after the viruses have been harvested and antigens have been prepared. Thus a process of the invention may include the step of mixing antigens from more than one influenza strain.

As described in reference 54, exemplary tetravalent vaccines can include hemagglutinin from two influenza A virus strains and two influenza B virus strains (A -A-B-B'), or from three influenza A virus strains and one influenza B virus strain ('A-A-A-B').

Influenza B virus currently does not display different HA subtypes, but influenza B virus strains do fall into two distinct lineages. These lineages emerged in the late 1980s and have HAs which can be antigenically and/or genetically distinguished from each other [55]. Current influenza B virus strains are either B/Victoria/2/87-like or B/Yamagata/16/88-like. Where a vaccine of the invention includes two influenza B strains, this will usually be one B/Victoria/2/87-like strain and one B/Yamagata/16/88-like strain. These strains are usually distinguished antigenically, but differences in amino acid sequences have also been described for distinguishing the two lineages *e.g.* B/Yamagata/16/88-like strains often (but not always) have HA proteins with deletions at amino acid residue 164, numbered relative to the 'Lee40' HA sequence [56].

Preferred A-A-B-B vaccines include hemagglutinins from; (i) a H1N1 strain; (ii) a H3N2 strain; (iii) a B/Victoria/2/87-like strain; and (iv) B/Yamagata/16/88-like strain.

In vaccines including three influenza A virus strains, these will usually be one H1 strain (*e.g.* a H1N1 strain) and two H3 strains (*e.g.* two H3N2 strains). The two H3 strains will have antigenically distinct HA proteins *e.g.* one H3N2 strain that cross-reacts with A/Moscow/10/99 and one H3N2 strain that cross-reacts with A/Fujian/41/2002. The two H3 strains may be from different clades

(clades A, B and C of H3N2 strains are disclosed in reference 57). In some embodiments, however, one of these strains (*i.e.* H1, or one of the two H3 strains) may be replaced by a pandemic strain.

Thus one preferred A-A-A-B vaccine includes hemagglutinins from: (i) a H1N1 strain; (ii) a A/Moscow/1 0/99-like H3N2 strain; (iii) a A/Fujian/41 1/2002-like H3N2 strain; and (iv) an influenza
5 B virus strain, which may be B/Victoria/2/87-like or B/Yamagata/ 16/88-1 like.

Another preferred A-A-A-B vaccine includes hemagglutinins from: (i) a H1N1 strain, (ii) a H3N2 strain, (iii) a H5 strain (*e.g.* a H5N1 strain) and (iv) an influenza B strain.

Another preferred A-A-A -B vaccine includes hemagglutinins from: (i) two different H1 strains, (ii) a H3N2 strain, and (iii) an influenza B strain.

10 Where antigens are present from two or more influenza B virus strains, at least two of the influenza B virus strains may have distinct hemagglutinins but related neuraminidases. For instance, they may both have a B/Victoria/2/87-like neuraminidase [58] or may both have a B/Yamagata/16/88-like neuraminidase. For instance, two B/Victoria/2/87-like neuraminidases may both have one or more of
15 the following sequence characteristics: (1) not a serine at residue 27, but preferably a leucine; (2) not a glutamate at residue 44, but preferably a lysine; (3) not a threonine at residue 46, but preferably an isoleucine; (4) not a proline at residue 51, but preferably a serine; (5) not an arginine at residue 65, but preferably a histidine; (6) not a glycine at residue 70, but preferably a glutamate; (7) not a leucine at residue 73, but preferably a phenylalanine; and/or (8) not a proline at residue 88, but preferably a glutamine. Similarly, in some embodiments the neuraminidase may have a deletion at residue 43, or
20 it may have a threonine; a deletion at residue 43, arising from a trinucleotide deletion in the NA gene, has been reported as a characteristic of B/Victoria/2/87-like strains, although recent strains have regained Thr-43 [58J]. Conversely, of course, the opposite characteristics may be shared by two B/Yamagata/16/88-1 like neuraminidases *e.g.* S27, E44, I 46, P51, R65, G70, L73, and/or P88. These amino acids are numbered relative to the 'Lee40' neuraminidase sequence [59]. Thus a A-A-B-B
25 vaccine of the invention may use two B strains that are antigenically distinct for HA (one B/Yamagata/16/88-like, one B/Victoria/2/87-like), but are related for NA (both B/Yamagata/16/88-like, or both B/Victoria/2/87-like).

In some embodiments, the invention does not encompass a trivalent split vaccine containing hemagglutinin from each of A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2 003 (H3N2) and
30 B/Jiangsu/10/2003 strains.

Strains whose antigens can usefully be included in the compositions include strains which are resistant to antiviral therapy (*e.g.* resistant to oseltamivir [60] and/or zanamivir), including resistant pandemic strains [61].

In some embodiments of the invention, a vaccine may include a small amount of mercury-based
35 preservative, such as thiomersal or merthiolate. When present, such preservatives will typically provide less than 5µg/ml mercury, and lower levels are possible *e.g.* <1µg/ml, <0.5µg/ml. Preferred

vaccines are free from **thiomersal**, and are more preferably mercury-free [23,62]. Such vaccines may include a non-mercurial preservative. Non-mercurial alternatives to thiomersal include **2-phenoxyethanol** or a-tocopherol succinate [23]. Most preferably, a vaccine is preservative-free.

In some embodiments, a vaccine may include a stabilising amount of gelatin *e.g.* at less than **0.1%**. In other embodiments, however, a vaccine is gelatin-free. The absence of gelatin can assure that the vaccine is safe in the small proportion of patients who are gelatin-sensitive [63,64].

In some embodiments, a vaccine may include one or more antibiotics *e.g.* neomycin, **kanamycin**, polymyxin B. In preferred embodiments, though, the vaccine is free from antibiotics.

In some **embodiments**, a vaccine may **include** formaldehyde. In preferred embodiments, though, the vaccine is free from **formaldehyde**.

As mentioned above, in some embodiments a vaccine may include egg components (*e.g.* ovalbumin and **ovomuroid**), but preferred embodiments are free from egg components.

The preparation of vaccines without the use of certain components and additives is disclosed in reference 65, thereby ensuring that these materials are not present even in residual amounts.

Hemagglutinin (HA) is the main **immunogen in current inactivated influenza vaccines, and vaccine** doses are standardised by reference to HA levels, **typically** measured by SRID. Existing vaccines **typically contain** about 15µg of HA per **strain**, although lower doses can be used *e.g.* for children, or in pandemic **situations**, or when using an adjuvant. Fractional doses such as ½ (*i.e.* **7.5µg** HA per strain), ¼ and ⅓ have been used, as have higher doses (*e.g.* 3x or 9x doses [66,67]). These vaccines have a dosage volume of 0.5ml *i.e.* a typical HA concentration of **30pg/ml/strain**. The trivalent INTANZA™ product contains 9µg of HA per strain in a 0.1ml volume *i.e.* a HA concentration of 90pg/ml/strain, giving a total HA concentration of **270µg/ml**.

Products of the present invention can **include** between **0.1** and **50µg** of HA per influenza strain per dose, preferably between **0.1** and 50µg *e.g.* 1~20µg. Ideally a product has ≤16µg hemagglutinin per strain *e.g.* 1-15µg, 1-10µg, 1-7.5µg, 1-5pg, *etc.* Particular HA doses per strain include *e.g.* about 15, about **10**, about 7.5, about 5, about 3.8, about 1.9, about 1.5, *etc.*

In surface antigen vaccines, HA can make up more than 50% (by mass) of total protein in the composition *e.g.* **between 60-100%, 60-90% or 60-80% of the total influenza protein in the immunogenic composition.**

For live vaccines, dosing is measured by median tissue culture infectious dose (TCID₅₀) rather than HA content *e.g.* a TCID₅₀ of between 10⁶ and 10⁸ (preferably between 10^{6.5}-10^{7.5}) per strain per dose.

Influenza strains used with the invention may have a natural HA as found in a wild-type virus, or a modified HA. For instance, it is known to modify HA to remove determinants (*e.g.* hyper-basic regions around the HA1/HA2 cleavage site) that cause a virus to be highly pathogenic in avian species. The use of reverse genetics facilitates such modifications.

Vaccine products of the invention can include components in addition to the influenza vaccine antigens. As discussed above, for example, they can **include** a biosoluble and biodegradable **matrix** material, or an oral film polymer.

Vaccine products may include a detergent. The level of detergent can vary widely *e.g.* between **0.01 - 5** 50µg detergent per µg of HA ('µg/µg') or between 0.05-50 µg. A low level of detergent can be used *e.g.* between 0.05-1µg/µg or between 0.1-1µg/µg, or a high level can be used *e.g.* between 5-30µg/µg. The detergent may be a single detergent (*e.g.* polysorbate 80, or CTAB) or a mixture (*e.g.* both polysorbate 80 **and** CTAB). Preferred detergents are non-ionic, such as polysorbate 80 ('Tween 80') or octyl phenol ethoxylate ('Triton X100'). Polysorbate 80 may be present at between
 10 **0.05-50** µg polysorbate 80 per µg of HA *e.g.* between 0.05-0.75µg/µg, 0.1-1 µg/µg, 0.1-0.8µg/µg, 0.1-0.5µg/µg, **5-40** µg/µg, 5-30µg/µg, or 8-25µg/µg. CTAB may be present at a low amount *e.g.* less than 0.1µg/µg, less than 0.05µg/µg.

As mentioned above, some vaccine products may include preservatives such as **thiomersal** or **2-phenoxyethanol**, but preferred vaccines are mercury- or preservative-free.

15 Vaccine products may include a physiological salt, such as a sodium salt. Sodium chloride (**NaCl**) is preferred, which may be present at between 1 and 20 **mg/ml**. **Other salts** that may be present include potassium chloride, potassium dihydrogen phosphate, **disodium** phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Vaccine products may include one or more buffers. Typical buffers include: a phosphate buffer; a
 20 **Tris** buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the **5-20mM** range.

Vaccine products are preferably sterile. Vaccine products are preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. **Vaccine products are preferably gluten-free.**

25 **Vaccine products can include immunostimulatory molecules. These can be mixed with antigen** before preparing a patch. Suitable classes of immunostimulatory molecule include, but are not limited to: TLR3 agonists; TLR4 agonists; TLR5 agonists; TLR7 agonists; TLR8 agonists; TLR9 agonists; and CD1d agonists. Suitable immunostimulatory molecules include, but are not limited to:
 30 **imidazoquinolines** such as imiquimod ('R-837') [68,69] and resiquimod ('R-848') [70], or salts thereof (*e.g.* the hydrochloride salts); aminoalkyl glucosaminide phosphate derivatives, such as RC-529 [71,72]; **a-glycosylceramides**, such as α-galactosylceramide; 'ER 804057' from reference 73; E5564 [74,75]; *etc.*

Methods for assessing antibody responses, neutralising capability and protection after influenza virus vaccination are well known in the art. Human studies have shown that antibody titers against
 35 **hemagglutinin** of human influenza virus are correlated with protection (a serum sample hemagglutination-inhibition titer of about 30-40 gives around 50% protection from infection by a

homologous virus) [76]. Antibody responses are typically measured by hemagglutination inhibition, by microneutralisation, by single radial immunodiffusion (SRID), and/or by single radial hemolysis (SRH). These assay techniques are well known in the art. Preferred vaccines satisfy 1, 2 or 3 of the CPMP criteria for efficacy. In adults (18-60 years), these criteria are: (1) $\geq 70\%$ seroprotection; (2) $\geq 40\%$ seroconversion; and/or (3) a GMT increase of ≥ 2.5 -fold. In elderly (>60 years), these criteria are: (1) $\geq 60\%$ seroprotection; (2) $\geq 30\%$ seroconversion; and/or (3) a GMT increase of ≥ 2 -fold. These criteria are based on open label studies with at least 50 patients.

Influenza vaccines are currently recommended for use in pediatric and adult immunisation, from the age of 6 months. Thus a human subject may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred subjects for receiving the vaccines are the elderly (e.g. ≥ 50 years old, ≥ 60 years old, and preferably ≥ 65 years), the young (e.g. ≤ 5 years old), hospitalised subjects, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, immunodeficient subjects, subjects who have taken an antiviral compound (e.g. an oseltamivir or zanamivir compound; see below) in the 7 days prior to receiving the vaccine, people with egg allergies and people travelling abroad. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population. For pandemic strains, administration to all age groups is preferred.

Administration of more than one dose (typically two doses) is particularly useful in immunologically naive patients e.g. for people who have never received an influenza vaccine before, or for vaccinating against a new HA subtype (as in a pandemic outbreak).

Bulk liquid vaccines

As mentioned above, the invention provides a bulk liquid vaccine comprising influenza virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least 12mg/ml and (b) the vaccine is substantially free from sucrose. These bulk vaccines can be useful intermediates during manufacture of vaccines for delivery by non-intramuscular routes. The HA concentration may be higher than 12mg/ml e.g. $>15\text{mg/ml}$, $>20\text{mg/ml}$, $>25\text{mg/ml}$, $>30\text{mg/ml}$, $>35\text{mg/ml}$, $>40\text{mg/ml}$, $>45\text{mg/ml}$, $>50\text{mg/ml}$.

The invention also provides a bulk liquid vaccine comprising an influenza A virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least 2mg/ml, (b) the hemagglutinin is not a H1 or a H3 hemagglutinin. Preferably, the vaccine (c) is substantially free from sucrose. These bulk vaccines, which contain neither H1 nor H3 influenza A virus hemagglutinin(s), can be useful intermediates during manufacture of vaccines for delivery by non-intramuscular routes. The HA concentration may be higher than 2mg/ml e.g. $>3\text{mg/ml}$, $>4\text{mg/ml}$, $>5\text{mg/ml}$, $>6\text{mg/ml}$, $\geq 7\text{mg/ml}$, $>8\text{mg/ml}$, $>9\text{mg/ml}$, $\geq 10\text{mg/ml}$, $\geq 12\text{mg/ml}$, $>14\text{mg/ml}$, $\geq 15\text{mg/ml}$, $\geq 16\text{mg/ml}$, $>18\text{mg/ml}$, $>20\text{mg/ml}$, $>25\text{mg/ml}$, $\geq 30\text{mg/ml}$, $>35\text{mg/ml}$, $>40\text{mg/ml}$, $>45\text{mg/ml}$, $>50\text{mg/ml}$. The hemagglutinin may be H5.

The invention also provides a bulk liquid vaccine comprising an influenza B virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least **2mg/ml**, (b) the hemagglutinin is not a **B/Shandong/7/97** hemagglutinin. Preferably, the vaccine (c) is substantially free from sucrose. These bulk vaccines, which contain neither HI nor H3 influenza A virus hemagglutinin(s), can be useful intermediates during manufacture of vaccines for delivery by non-intramuscular routes. The HA concentration may be higher than **2mg/ml e.g. $\geq 3\text{mg/ml}$, $\geq 4\text{mg/ml}$, $> 5\text{mg/ml}$, $> 6\text{mg/ml}$, $> 7\text{mg/ml}$, $\geq 8\text{mg/ml}$, $\geq 9\text{mg/ml}$, $\geq 10\text{mg/ml}$, $> 12\text{mg/ml}$, $> 14\text{mg/ml}$, $\geq 15\text{mg/ml}$, $> 16\text{mg/ml}$, $\geq 18\text{mg/ml}$, $> 20\text{mg/ml}$, $> 25\text{mg/ml}$, $> 30\text{mg/ml}$, $> 35\text{mg/ml}$, $\geq 40\text{mg/ml}$, $> 45\text{mg/ml}$, $> 50\text{mg/ml}$.**

The invention also provides a bulk liquid vaccine comprising an influenza B virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least **2mg/ml**, (b) the influenza B virus is a **B/Yamagata/16/88-like** strain. Preferably, the vaccine (c) is substantially free from sucrose. These bulk vaccines, which contain neither HI nor H3 influenza A virus hemagglutinin(s), can be useful intermediates during manufacture of vaccines for delivery by non-intramuscular routes. The HA concentration may be higher than **2mg/ml e.g. $> 3\text{mg/ml}$, $\geq 4\text{mg/ml}$, $> 5\text{mg/ml}$, $> 6\text{mg/ml}$, $> 7\text{mg/ml}$, $> 8\text{mg/ml}$, $> 9\text{mg/ml}$, $> 10\text{mg/ml}$, $> 12\text{mg/ml}$, $> 14\text{mg/ml}$, $> 15\text{mg/ml}$, $\geq 16\text{mg/ml}$, $\geq 18\text{mg/ml}$, $> 20\text{mg/ml}$, $> 25\text{mg/ml}$, $> 30\text{mg/ml}$, $> 35\text{mg/ml}$, $> 40\text{mg/ml}$, $> 45\text{mg/ml}$, $\geq 50\text{mg/ml}$.**

The invention also provides a bulk liquid vaccine comprising hemagglutinin from at least two strains of influenza virus, wherein (a) the hemagglutinin concentration is at least **2mg/ml/strain** and (b) the vaccine is substantially free from sucrose. The HA concentration may be higher than **2mg/ml/strain e.g. $> 3\text{mg/ml/strain}$, $> 4\text{mg/ml/strain}$, $> 5\text{mg/ml/strain}$, $> 6\text{mg/ml/strain}$, $> 7\text{mg/ml/strain}$, $\geq 8\text{mg/ml/strain}$, $> 9\text{mg/ml/strain}$, $> 10\text{mg/ml/strain}$, $\geq 12\text{mg/ml/strain}$, $> 14\text{mg/ml/strain}$, $\geq 15\text{mg/ml/strain}$, $> 16\text{mg/ml/strain}$, $\geq 18\text{mg/ml/strain}$, $> 20\text{mg/ml/strain}$, $> 25\text{mg/ml/strain}$, $> 30\text{mg/ml/strain}$, $> 35\text{mg/ml/strain}$, $> 40\text{mg/ml/strain}$, $> 45\text{mg/ml/strain}$, $\geq 50\text{mg/ml/strain}$. This sucrose-free bulk multivalent vaccine is ideally 2-valent, 3-valent or 4-valent. In a 3-valent vaccine, therefore, the total HA concentration is at least **6mg/ml****

The HA concentration in these bulk liquid vaccines is preferably a HA concentration as measured by SRID *e.g.* using regulator)ly-approved reference reagents. SRID can be performed on bulk vaccine prior to its formulation (*e.g.* prior to coating or to film formation), but in some embodiments it is performed on material recovered from an already-formulated product (*e.g.* after recovering antigen from a coated needle). An advantage of SRID is that it can distinguish between different HAs in a single sample *e.g.* each HA in a trivalent vaccine can be analysed in parallel. Where a composition includes a particular HA, therefore, its content can be assayed even in the presence of other HAs.

In addition to being substantially free from sucrose, these bulks may be substantially free from mannitol. The bulks may even be substantially free from sucrose, trehalose, maltose, lactulose, lactose, cellobiose, sorbitol, mannitol, maltitol, erythritol and xylitol. In some embodiments, the bulks are substantially free from disaccharide and from sugar alcohol.

General

The term "**comprising**" encompasses "including" as well as "consisting" *e.g.* a composition "comprising" X may consist exclusively of X or may include something additional *e.g.* X + Y.

The word "substantially" does not exclude "completely" *e.g.* a composition which is "substantially free" from Y may be completely free from Y. Where necessary, the word "substantially" may be omitted from the definition of the invention.

The term "about" in relation to a numerical value *x* is optional and means, for example, $x \pm 5\%$.

Unless specifically stated, a process comprising a step of mixing two or more components does not require any specific order of mixing. Thus components can be mixed in any order. Where there are three components then two components can be combined with each other, and then the combination may be combined with the third component, *etc.*

Where animal (and particularly bovine) materials are used in the culture of cells, they should be obtained from sources that are free from transmissible spongiform encephalopathies (**TSEs**), and in particular free from bovine spongiform encephalopathy (BSE). Overall, it is preferred to culture cells in the total absence of animal-derived materials.

The invention provides lyophilisation-free concentration procedures. This means that the invention uses antigen which is concentrated and formulated without being lyophilised. The scope of protection is not intended to exclude situations where a lyophilised antigen is added to a vaccine, provided that at least some of the vaccine antigen is being concentrated and formulated **without** being lyophilised. Thus the use of a lyophilisate while also performing a lyophilisation-free concentration procedure of the invention does not fall outside the intended scope of overall protection.

Where a compound is administered to the body as part of a composition then that compound may alternatively be replaced by a suitable prodrug.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows SEC chromatographs of a monovalent influenza B virus bulk antigen. The bulk was analysed (A) before lyophilisation, (B) after lyophilisation in the presence of sucrose, or (C) after lyophilisation in the presence of sucrose and mannitol. The x-axis shows retention time (minutes) and the y-axis shows absorbance units.

Figure 2 shows serum HI titers for (A) H1N1 Brisbane (B) H3N2 Brisbane (C) B Florida. The 4 pairs of bars are, from left to right: **trivalent unadjuvanted; trivalent adjuvanted; monovalent unadjuvanted; and PBS negative control.** The left-hand bar in each pair is **un-concentrated** monovalent bulk; the right-hand bar is **TFF-concentrated** antigen.

MODES FOR CARRYING OUT THE INVENTION***Impact of lyophilisation on influenza virus hemagglutinin***

Monovalent influenza virus bulk antigen was analysed by size exclusion chromatography (SEC), and then analysed again after lyophilisation and reconstitution. Two different lyophilisation procedures were tested: one with sucrose alone as tyoprotectant, and one with both sucrose and mannitol. The results in Figure I show that a prominent peak at 5 minutes (Figure 1A) has disappeared in the reconstituted materials (Figures IB & IC). Thus antigen concentration should avoid lyophilisation because this treatment tangibly alters the vaccine antigen.

Comparison of antigen concentration techniques

The starting HA concentration was about 0.5mg/ml in monovalent influenza virus bulks. Three different methods were used for concentration of various bulks; centrifugal filtration; ultrafiltration; and TFF. Centrifugal filtration used a Millipore™ device with a 10kDa cut-off, operated at 5000rpm for 45 minutes. Ultrafiltration used an Amicon™ stir cell concentrator with a 10kDa cut-off membrane made from regenerated cellulose, operated under pressurised nitrogen for 1 hour. TFF used a modified polyethersulfone hollow fiber filter with a 30kDa cut-off, operated to concentrate a 45ml starting volume down to 1.5ml. To increase the concentration which could be achieved the centrifugation and ultrafiltration methods were also combined with lyophilisation.

Centrifugal filtration achieved a concentration of -10-fold on its own. If followed by lyophilisation, additional SEC peaks appeared and the reconstituted samples contained visible aggregates.

Ultrafiltration followed by lyophilisation, and then reconstitution back into the starting volume, gave material which had a HA concentration (as measured by SRID) comparable to the starting material, indicating no loss of functional antigen. The reconstituted material was stable for >2 weeks.

Whereas these two methods required lyophilisation to achieve a high concentration (e.g. 30-fold), this could be achieved in a single step when using TFF. Moreover, the concentrated material is stable for >2 weeks. To achieve a high antigen concentration without requiring lyophilisation, therefore, TFF is the preferred technique.

TFF and lyophilisation

Monovalent bulks of three egg-grown strains of influenza A and B viruses were treated by: (i) addition of sucrose; (ii) lyophilisation in the presence of sucrose, with reconstitution in the original volume; (iii) the same lyophilisation as in (ii), but with reconstitution in half of the original volume; (iv) concentration by TFF; or (v) concentration by TFF as in (v), followed by lyophilisation and reconstitution as in (ii). In each case HA content was assessed by SRID and is shown below as the absolute value and also as a concentration factor relative to the respective starting bulks (which had a HA concentration ranging from 311 to 437 µg/ml). If the concentration factor in (i) or (ii) is less than 1, the lyoprotectant and/or lyophilisation is interfering with HA binding; the same applies if the factor in (iii) is less than 2, or if the factor in (v) is less than in (iv).

Results from one set of experiments were as follows:

Strain	Treatment	HA (mg/ml)	Factor
1	(i)	0.304	0.70
	(ii)	0.168	0.38
	(iii)	0.721	1.65
	(iv)	20.691	47.35
	(v)	19.616	44.89
2	(i)	0.314	0.98
	(ii)	0.117	0.37
	(iii)	0.393	1.23
	(iv)	8.935	27.92
	(v)	10.041	31.38
3	(i)	0.247	0.79
	(ii)	0.123	0.40
	(iii)	0.413	1.33
	(iv)	5.742	18.46
	(v)	5.235	16.83

In all cases, therefore, treatments (i) to (iii) led to a reduction in detectable HA, although the effect of treatment (i) of strain 2 was very small. Moreover, although addition of the lyoprotectant alone led to a reduction in HA, the **post-reconstitution** reduction was even greater *i.e.* the reduction in HA levels was always greater after treatment (ii) than treatment (i). Thus the lyophilisation leads to a large drop in detectable HA. With the exception of strain 2, a similar effect was seen after TFF *i.e.* the lyophilisation reduced the SRID-detectable HA content.

Results from a separate set of experiments, using only treatments (iv) and (v), were as follows:

Strain	Treatment	HA (mg/ml)	Factor
1	(iv)	29.05	59x
	(v)	27.54	56x
2	(iv)	28.07	82x
	(v)	24.34	71x
3	(iv)	15.30	61x
	(v)	10.98	44x

10

Thus TFF alone can achieve a **>50-fold** concentration. As seen above, however, the additional step of **lyophilisation** always led to a **reduction** in SRID-measured HA content.

Immunogenicity studies

Influenza monovalent bulks from the 2008/2009 season were concentrated by TFF. The concentrated influenza antigens were evaluated for their immunogenicity *in vivo* and compared against the original monovalent bulk materials. Antigens were administered intramuscularly to female **Balb/C** mice using
 5 2 immunizations separated by 3 weeks. Antigens were administered as monovalent vaccines (0.3µg HA), as trivalent vaccines (0.3µg HA), or as adjuvanted trivalent vaccines (0.3µg HA with **MF59 oil-in-water emulsion** adjuvant). PBS was used as a negative control. Functional anti-influenza antibody titers were assessed by hemagglutination inhibition (HI).

The influenza monovalent bulks were concentrated by TFF to 29, 28 and 20 **mg/ml** HA. All three
 10 concentrates had a HA to total protein ratio in the range of 0.6 - 0.8 (µg of HA per µg of protein). Tween 80 (polysorbate 80) was concentrated along with the antigen and was estimated as **8-14mg/ml**. The ratio of Tween 80 to HA for the concentrated antigens was 0.1-0.2 µg per µg of HA, which is the same as in typical monovalent bulks. In contrast to Tween 80, CTAB did not become concentrated during the process and was well below the level which was present in the unprocessed
 15 monovalent bulks (<**0.01** µg detergent per µg of HA). The concentrated antigens were stable by **SRID** for 3-4 weeks in solution at **4°C**.

HI titers 2-weeks after the 2nd immunization are shown in Figure 2. For all three strains (H1N1 Brisbane; **H3N2** Brisbane; B Florida) the immunogenicity of the concentrated antigens was comparable to the non-concentrated monovalent bulks.

20 It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

REFERENCES

- [1] Malke *et al.* (2010) *The Internet Journal of Pediatrics and Neonatology*. Vol **11**, number 2.
- [2] Koutsonanos *et al.* (2009) *PLoS ONE* 4(e): **e4773**.
- [3] Quan *et al.* (2009) *PLoS ONE* 4(9):e?1 52.
- [4] **WO2007/030477**.
- [5] **US-6945952**.
- [6] **US-721 1062**.
- [7] **US-7 182747**.
- [8] Oh *et al.* (2006) American Association of Pharmaceutical Scientists, 2006 Annual Meeting and Exposition. *The AAPS Journal*. **8(S2)**. (Annual Meeting).
- [9] Gill & Prausnitz (2007) *J Control Release* 117:227-37.

- [10] WO2007/124393.
- [11] WO2007/061964.
- [12] WO2007/059289.
- [13] Jin *et al.* (2009) *Biomed Microdevices*. 11(6): 1195-203.
- [14] Su *et al.* (2009) *ACS Nano*. 3(11):3719-29.
- [15] Cui & Mumper (2002) *Pharmaceutical Research* 19:947-53.
- [16] WO02/051382.
- [17] WO 2010/002418.
- [18] *Vaccines*, (eds. Plotkin & Orenstein). 4th edition, 2004, ISBN: 0-7216-9688-0.
- [19] WO02/28422.
- [20] WO02/067983.
- [21] WO02/074336.
- [22] WO01/21151.
- [23] WO02/097072.
- [24] WO2005/113756.
- [25] Huckriede *et al.* (2003) *Methods Enzymol* 373:74-91.
- [26] W096/37624.
- [27] W098/46262.
- [28] W095/18861.
- [29] Bright *et al.* (2008) *PLoS ONE* 3:e150.
- [30] Crevar & Ross (2008) *Virology Journal* 5:131.
- [31] WO97/37000.
- [32] Brands *et al.* (1999) *Dev Biol Stand* 98:93-100.
- [33] Halperin *et al.* (2002) *Vaccine* 20:1240-7.
- [34] Tree *et al.* (2001) *Vaccine* 19:3444-50.
- [35] Kistner *et al.* (1998) *Vaccine* 16:960-8.
- [36] Kistner *et al.* (1999) *Dev Biol Stand* 98:101-110.
- [37] Bruhl *et al.* (2000) *Vaccine* 19:1149-58.
- [38] Pau *et al.* (2001) *Vaccine* 19:2716-21.
- [39] WO03/076601.
- [40] WO2005/042728.
- [41] WO03/043415.
- [42] WO01/85938.
- [43] WO2006/108846.
- [44] EP-A-1260581 (WO01/64846).
- [45] WO2006/071563.
- [46] WO2005/113758.
- [47] WO2006/027698.
- [48] WO03/023021.
- [49] WO03/023025.
- [50] WO97/37001.
- [51] EP-B-0870508.
- [52] US 5948410.

- [53] WO2007/052163.
- [54] WO2008/06863 I.
- [55] Rota *et al.* (1992) *J Gen Virol* 73:2737-42.
- [56] GenBank sequence GI:325 176.
- [57] Holmes *et al.* (2005) *PLoS Biol.* 3(9):e300.
- [58] McCullers *et al.* (1999) *J Virol* 73:7343-8.
- [59] GenBank sequence GI:325237.
- [60] Herlocher *et al.* (2004) *J Infect Dis* 190(9):1627-30.
- [61] Le *et al.* (2005) *Nature* 437(7062): 1 108.
- [62] BanzhofT(2000) *Immunology Letters* 71:91-96.
- [63] Lasley (2007) *Pediatric Asthma, Allergy & Immunology.* 20(3): 201-5.
- [64] Coop *et al.* (2008) *Int Arch Allergy Immunol.* 146(1):85-8.
- [65] WO2009/00121 7
- [66] Treanor *et al.* (1996) *J Infect Dis* 173:1467-70.
- [67] Keitel *et al.* (1996) *Clin Diagn Lab Immunol* 3:507-10.
- [68] US 4,680,338.
- [69] US 4,988,815.
- [70] W092/I5582.
- [71] Johnson *et al.* (1999) *Bioorg Med Chem Lett* 9:2273-2278.
- [72] Evans *et al.* (2003) *Expert Rev Vaccines* 2:219-229.
- [73] WO03/OI 1223.
- [74] Wong *et al.* (2003) *J Clin Pharmacol* 43(7):735-42.
- [75] US2005/02155 17.
- [76] Potter & Oxford (1979) *Br Med Bull* 35: 69-75.

CLAIMS

1. A process for preparing a vaccine, comprising steps of (i) increasing the concentration of an antigen in a liquid composition including that antigen, to provide a concentrated antigen, and (ii) formulating a vaccine from the concentrated antigen; wherein the concentrated antigen is not lyophilised between or during steps (i) and (ii).
2. The process of claim 1, wherein the vaccine is to protect against disease caused by a bacterium, a virus, a fungus, and/or a parasite.
3. The process of claim 2, wherein the vaccine is an influenza vaccine.
4. The process of claim 3, which is a process for preparing an influenza vaccine, comprising steps of (i) increasing the concentration of an influenza virus hemagglutinin in a liquid composition including that hemagglutinin, to provide a concentrated antigen, and (ii) formulating an influenza vaccine from the concentrated antigen.
5. The process of claim 4, which is a process for preparing a vaccine comprising hemagglutinin from n different influenza virus strains, comprising steps of (i) increasing the concentration of influenza virus hemagglutinin in n separate liquid compositions, each including hemagglutinin from a different strain, to provide n separate concentrated antigens, and (ii) formulating a multivalent influenza vaccine from the n separate concentrated antigens; wherein none of the n separate concentrated antigens is lyophilised between or during steps (i) and (ii).
6. The process of any preceding claim, wherein the liquid composition is substantially free from exogenous sugar alcohols and/or from exogenous disaccharides.
7. The process of any preceding claim, wherein antigen concentration is increased in step (i) by centrifugal filtration, by ultrafiltration, or by tangential flow filtration.
8. The process of claim 7, wherein antigen concentration is increased in step (i) by tangential flow filtration
9. The process of any preceding claim, wherein step (i) increases antigen concentration by at least 10-fold.
10. The process of any preceding claim, wherein the liquid composition includes a detergent.
11. The process of claim 10, wherein the detergent is an ionic detergent (e.g. CTAB) or a non-ionic detergent (e.g. polysorbate 80).
12. The process of claim 10 or claim 11, wherein the detergent is concentrated in step (i).
13. The process of claim 12, wherein the detergent is concentrated to a degree less than the degree to which the antigen is concentrated.
14. The process of claim 10 or claim 11, wherein the detergent is not concentrated in step (i).
15. The process of any preceding claim, wherein step (ii) prepares a solid vaccine form.

16. The process of claim 15, wherein step (ii) involves drying by evaporation.
17. The process of any preceding claim, wherein step (ii) prepares solid biodegradable microneedles from the concentrated antigen.
18. The process of any one of claims 1 to 16, wherein step (ii) coats solid microneedles with the concentrated antigen.
19. The process of claim 18, wherein the microneedles are metal or plastic.
20. The process of claim 18 or claim 19, wherein step (ii) applies the concentrated antigen to the surface of one or more solid microneedles to provide a coated microneedle device for injection of the vaccine.
21. The process of any one of claims 17 to 20, wherein the microneedles are **100-2500** μm long
22. The process of any one of claims 1 to 16, wherein step (ii) prepares a thin film from the concentrated antigen.
23. The process of claim 22, wherein step (ii) comprises mixing the concentrated antigen with one or more orally-soluble polymers, then forming a film using the mixture to provide a thin film suitable for buccal administration of the vaccine.
24. The process of claim 22, wherein step (ii) comprises mixing the concentrated vaccine antigen with one or more topically-soluble polymers, then forming a film using the mixture to provide a thin film suitable for transcutaneous administration of the vaccine.
25. The process of any one of claims 22 to 24, wherein the film is **10-500** μm (*e.g.* **75-150** μm) thick.
26. The process of any one of claims 22 to 25, wherein the antigen is encapsulated inside microparticles within the film.
27. A process for preparing a packaged vaccine, comprising: (i) preparing a solid vaccine by the process of any one of claims 15 to 26; then (ii) packaging a solid vaccine into an individual unit dose pouch.
28. The process of any preceding claim, wherein the antigen comprises an influenza hemagglutinin, and wherein hemagglutinin content is measured before the formulating step by using **SRID**.
29. The process of any preceding claim, wherein the antigen comprises an influenza hemagglutinin, and wherein hemagglutinin content is measured after the formulating step by using **SRID**.
30. A vaccine prepared by the process of any preceding claim.
31. A method of raising an immune response in a subject, comprising the step of administering the vaccine of claim 30 to the subject.
32. A liquid vaccine comprising influenza virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least **12mg/ml** and (b) the vaccine is substantially free from sucrose.

33. A liquid vaccine comprising an influenza A virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least **2mg/ml**, (b) the hemagglutinin is **not a H1** or a **H3** hemagglutinin.
34. A liquid vaccine comprising an influenza **B** virus hemagglutinin, wherein (a) the hemagglutinin concentration is at least 2mg/ml, (b) the influenza B virus is a **B/Yamagata/16/88-like** strain.
- 5 35. The liquid vaccine of claim 33 or claim 34, wherein the vaccine is substantially free from sucrose.
36. A liquid vaccine comprising hemagglutinin from at least two strains of influenza virus, wherein (a) the hemagglutinin concentration is at least 2mg/ml/strain and (b) the vaccine is substantially free from sucrose.
- 10 37. The liquid vaccine of claim 32, claim 35 or claim 36, which is substantially free from **mannitol**.
38. The liquid vaccine of claim 32, claim 35, claim 36 or claim 37, which is substantially free from disaccharide and from sugar alcohol.

FIGURE 1

FIGURE 1A

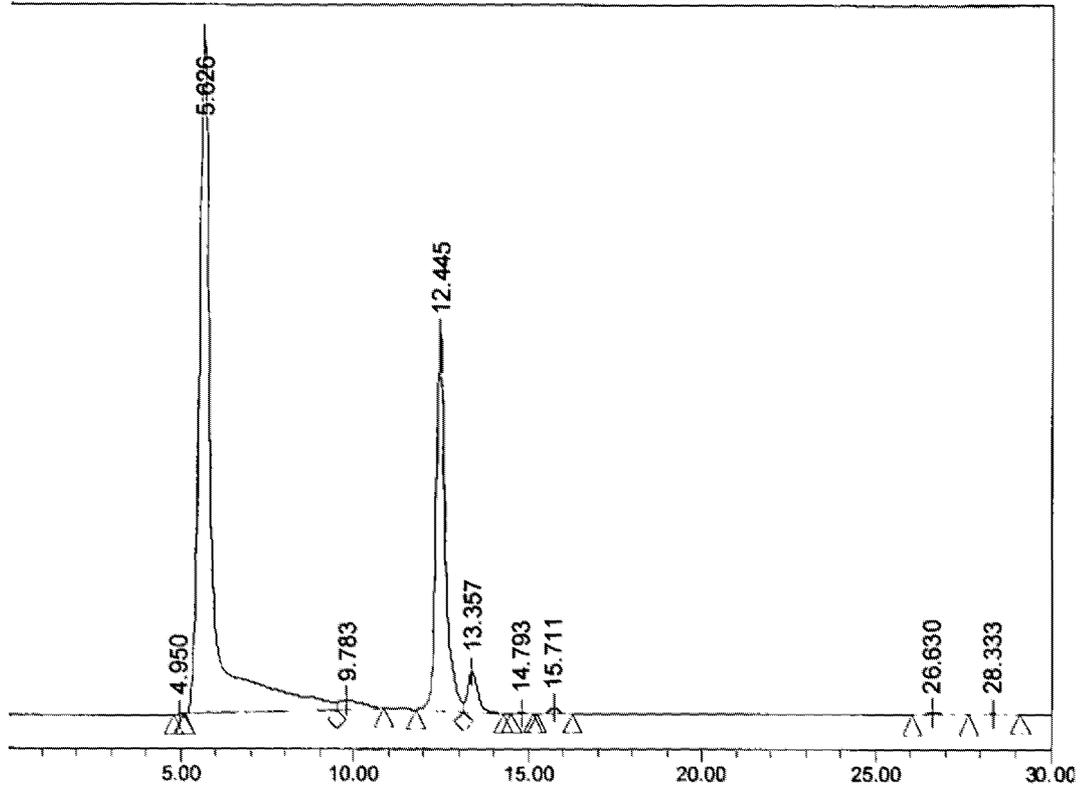


FIGURE 1B

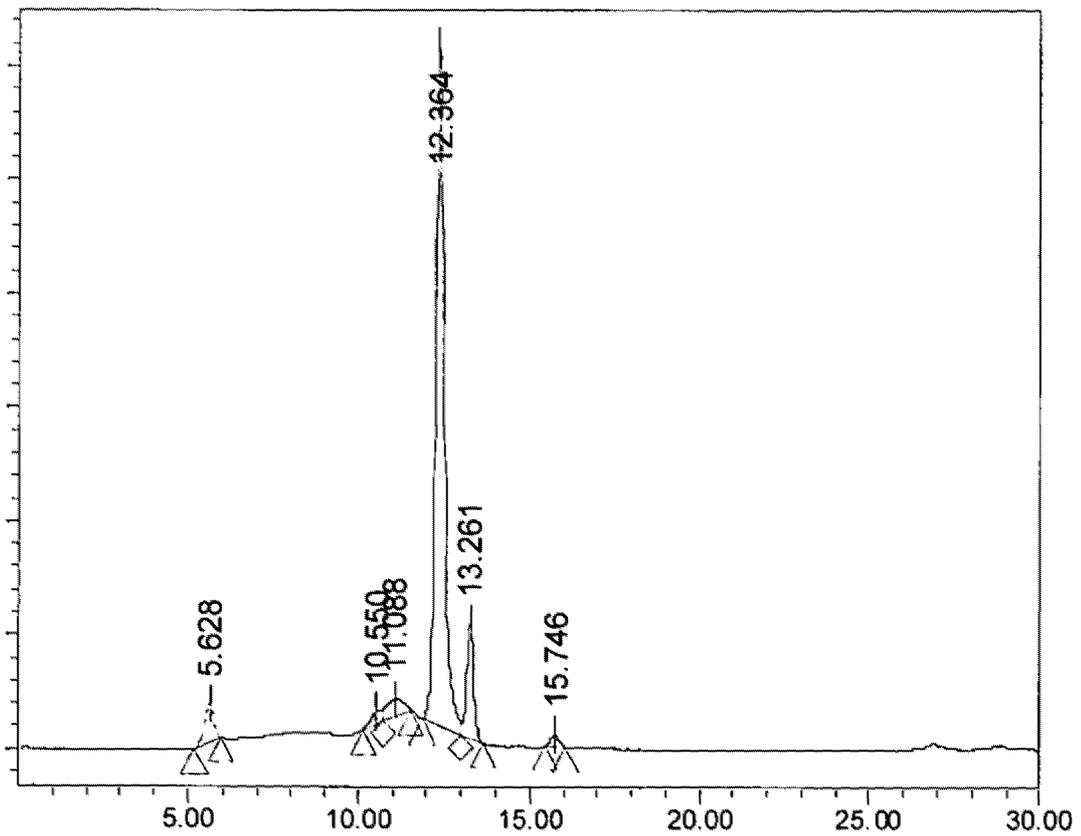


FIGURE 1C

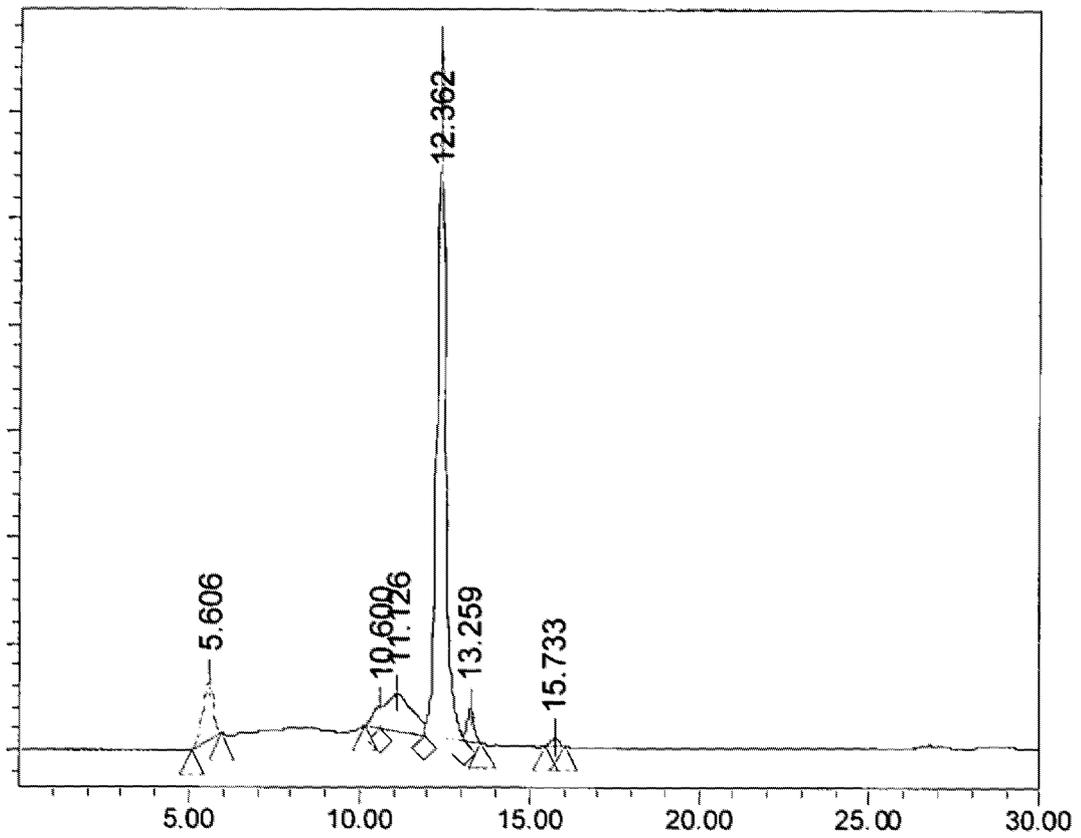


FIGURE 2

FIGURE 2A

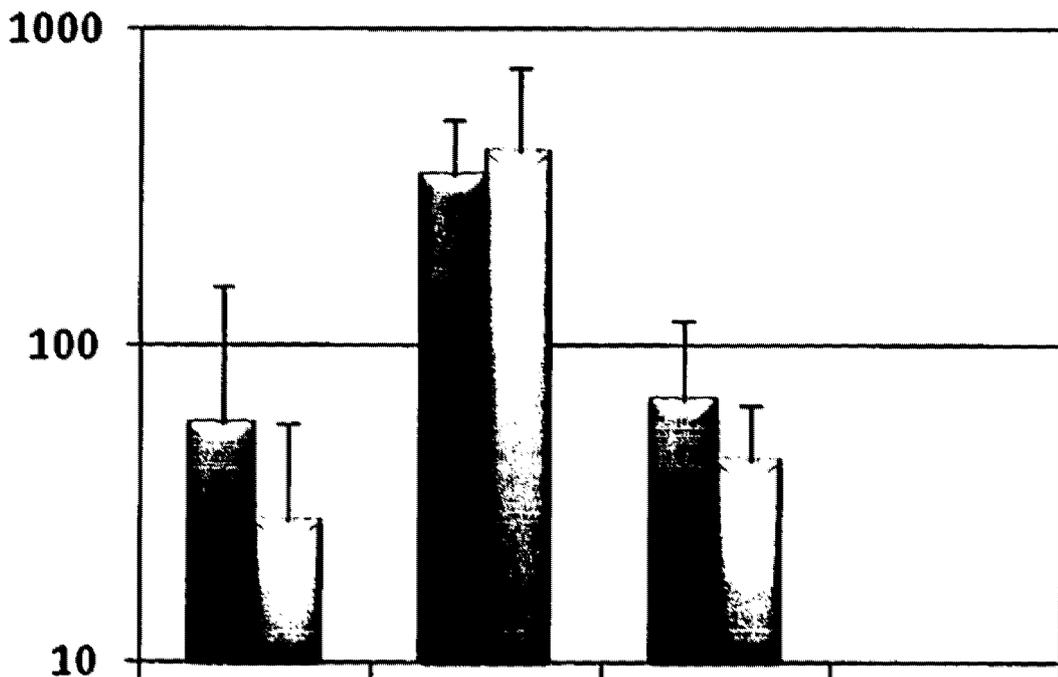


FIGURE 2B

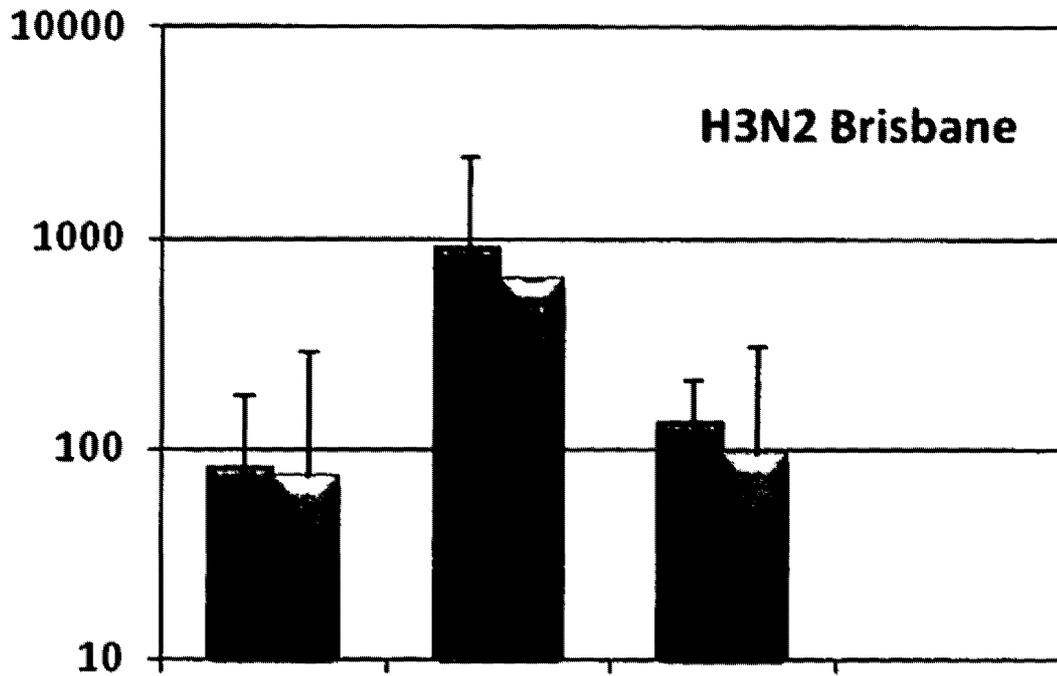


FIGURE 2C

